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Identification of New Compounds in *Epimedium* L. based on Flavonol Secondary Metabolism and High-Resolution Mass Spectrometry

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Summary: To derive and verify the chemical structure of the new components in *Epimedium*, the laws of secondary metabolism and high-resolution mass spectrometry (HRMS) were combined. Based on the chemical literature of *Epimedium*, the secondary metabolism network of flavonols was constructed, and the possible metabolites were deduced. After the metabolites, information was imported into PeakView software, and the ions with a mass error < 5 ppm, correct isotope distribution, and containing secondary fragments were taken as the target compounds. The chemical structures of new compounds were identified and verified by combining Formula Finder, Mass Calculators, online databases (SciFinder, Reaxys, ChemSpider, etc.) and secondary fragmentation rules. In this study, a total of 4 metabolic pathways and 64 compound structures were deduced, and two new components and 12 new compounds were identified in 54 batches of *Epimedium* samples from 15 species by high-resolution mass spectrometry. Furthermore, the long and tedious steps of phytochemical separation were simplified, experimental costs were reduced, and a new idea and method were suggested for the analysis and identification of secondary metabolites with pharmacological activity.

Keywords: *Epimedium*; New compound; Secondary metabolism; High-resolution mass spectrometry; Flavonol.

Introduction

Epimedium Folium is the dry aboveground part of the *Epimedium* L., a perennial herb in Berberidaceae family [1-2]. This genus is mainly distributed in China, North Africa, and North Korea. There are about 55 species in the whole genus, among which 47 species are found in China [3-5]. According to Chinese Pharmacopoeia (2015 edition), there are 5 species, including *Epimedium brevicornu* Maxim., *Epimedium wushanense* T.S Ying, *Epimedium sagittatum* (Sieb. Et Zucc.) Maxim., and 50 Chinese patent medicines containing Epimedium Folium [6]. Recent studies have shown that Epimedium Folium possesses multiple pharmacodynamic functions, including the promotion of immunity, reproduction, cardio-cerebrovascular system, as well as an anti-aging effect [7-15]. The main active components of Epimedium Folium are flavones derivatives with 2-phenyl chromogen as the parent nucleus, with isopentenyl, hydroxyl, glycosyl, and methoxy groups at C-8, C-7, C-5, C-3, C-4' sites [16-21]. Sixty-two compounds, including icariin, epimedin, and baohuoside, have been reported so far. [22-26]. There has been no systematic report on the biosynthetic pathway of flavonoids in *Epimedium*. Previously, Zhang *et al* indicated that icariin and epimedin are a result of enzymatic reactions with flavonol as the substrate; these types of enzymatic reactions mainly include isopentenyl substitution, acylation, and methylation or transglycation radical reaction [27]. Among them, the glycosylation of the active hydroxyl group at the C-3 position of parent nucleus is particularly important. So far, only a few studies reported on the secondary metabolic enzymes and structural genes of epimedium flavonoids. Yamamoto *et al* preformed biochemical analysis on dimethylallyl transferase [28]. By reviewing and reorganizing the epimedium chemistry-related literature, we initially constructed a secondary metabolic network containing the prenyl flavonol component, deduced four metabolic pathways, and 64 compound structures based on an in-depth analysis of its biosynthesis laws. We finally identified two new components and 12 new compounds with HRMS analysis. The results not only verified the correctness of the constructed secondary metabolic pathway but also laid a scientific foundation for the subsequent regulation of secondary metabolism.

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Experimental

Materials and Methods

Instruments and materials

LC-30A ultra-high performance liquid chromatography with a binary high-pressure pump, automatic sampler, and column compartment was purchased from Shimadzu Corporation (Japan). Triple TOF™ 4600 quadrupole tandem time-of-flight high-resolution mass spectrometer was obtained from AB Corporation (USA). JY92-Ⅱ D ultrasonic cell crusher was bought from Ningbo Xinyi Ultrasonic Equipment Co., Ltd., China; BJ-100 ultra-high-speed Chinese medicine grinder was purchased from Deqing Baijie Electric Co., Ltd., China; VGT-2013QT type ultrasonic cleaning machine was acquired from Goode Ultrasonic Company, China. BSA224S-CW type 1/10000 analytical balance was purchased from Sartorius (Germany).

Reference substance (purity > 98%): icariin (Lot No.: 489-32-7), icariside I (Lot No.: 56725-99-6), icariside II (Lot No.: 113558-15-9), epimedin A (Lot No.: 110623-72-8), epimedin B (Lot No.: 110623-73-9), epimedin C (Lot No.: 110642-44-9), sagittatoside A (Lot No.: 118525-35-2), sagittatoside B (Lot No.: 118525-36-3), 2''-O-rhamnosylicariside II (Lot No.: 135293-13-9), baohuoside II (Lot No.: 55395-07-8), epimedoside A (Lot No.: 39012-04-9), desmethylicaritin (Lot No.: 28610-31-3), baohuoside V (Lot No.: 118544-18-6) and acuminatoside (Lot No.: 143601-07-4) were purchased from Chengdu Efa Biotechnology Co., Ltd (China). HPLC grade Acetonitrile (Merck Germany) and formic acid (ACS, USA) were of chromatographic grade, and the remaining reagents were of analytical grade. Samples of different species of *Epimedium* were collected from Chongqing, Sichuan, Hubei, Hunan, etc., and were identified as the above-ground part of the perennial herbaceous plant *Epimedium* by the associate researcher Liu Xiang of Chongqing Academy of Chinese Meteria Medica. The information of 54 batches of samples is shown in Table-1.

Chromatography and mass spectrometry conditions

The ACE Excel 3 Super C_{18} column (10 mm × 2.1 mm, 3.0 μm) was used. The column temperature was 35 ºC; the mobile phase was acetonitrile (A) - 0.1% formic acid (B) aqueous solution; the flow rate was 0.2 mL/min; gradient elution: 0 ~ 1.0 min, 17% A; 1.0 ~ 11.0 min, 17% ~ 80% A; 11.0 ~ 12.0 min, 80% A; 12.0 ~ 12.1 min, 80% ~ 17% A; 12.1 ~ 15.0 min, 17% A. The injection volume was 2 μL.

Electrospray ion source (ESI), data collection was in positive ion mode. Spray voltage (IS) was +5500 V; atomizing gas pressure (GS1): 0.37 MPa; air curtain gas pressure (CUR): 0.16 MPa; auxiliary gas pressure (GS2): 0.37 MPa; ion source temperature (TEMP): 600 ºC; cluster cracking voltage (DP): 60 V; collision energy (CE): 45 V; collision energy rolling interval (CES): 15 V; detection mode was IDA (Information related acquisition mode). Moreover, the condition of triggering level 2 was multi-quality deficit (MMDF) and dynamic background deduction (DBS), which is the priority for level 2 scanning.

Table-1: *Epimedium* sample collection information table.

Scientific name	Sample No.	Collection location
***Epimedium baojingense* Q.L. Chen et B.M. Yang**	**S1-S2**	**Hunan**
***Epimedium mikinorii* Stearn**	**S3-S4**	**Hubei**
***Epimedium leptorrhizum* Steam**	**S5-S6**	**Chongqing**
***Epimedium sutchuenense* Franch.**	**S7-S8**	**Chongqing, Sichuan**
***Epimedium truncatum* H. R. Liang**	**S9-S10**	**Hunan**
***Epimedium hunanense* Hand.-Mazz.**	**S11-S15**	**Hunan**
***Epimedium pubescens* Maxim.**	**S16-S22**	**Sichuan, Chongqing**
***Epimedium membranaceum* K.Meyer**	**S23-S24**	**Sichuan**
***Epimedium myrianthum* Stearn**	**S25-S41**	**Chongqing**
***Epimedium dolichostemon* Stearn**	**S42-S44**	**Chongqing**
***Epimedium borealiguizhouense* S. Z. He et Y. K. Ying**	**S45-S46**	**Guizhou**
***Epimedium acuminatum* Franch.**	**S47-S49**	**Chongqing**
***Epimedium jinchengshanense* Yan J.Zhang & J.Q.Li**	**S50**	**Chongqing**
***Epimedium simplici folium* Ying**	**S51-S52**	**Chongqing**
***Epimedium zhushanense* K.F. Wu et S.X. Qian**	**S53-S54**	**Chongqing**

Sample solution preparation

Epimedium sample powder (0.1 g) was placed in a 5 mL tube, mixed with 2 mL of 50% EtOH, after which the ultrasonic extraction (power: 600 W) was performed for 30 min. The extraction was filtered, after which, 1mL was transferred into a 10 mL volumetric bottle containing 9ML of chromatographic methanol (total volume to 10mL). The samples were filtered with a 0.22 μm microporous membrane before entering the chromatograph.

Data analysis

The relevant literature on *Epimedium* was reviewed to summarize the secondary metabolic laws of the flavonoids in *Epimedium*. The possible metabolites were derived according to its unique biosynthetic pathway, and the product information was substituted into PeakView software to construct a new compound screening database. The data from different samples collected by Q-TOF were extracted and analyzed, and the ions with a mass error < 5 ppm, with correct isotope distribution, and containing secondary fragments were taken as the target compounds. The chemical structures of new compounds were identified and verified by combining Formula Finder, Mass Calculators, online databases (SciFinder, Reaxys, ChemSpider, Metlin, and HMDB) and secondary fragmentation rules.

Results and Discussion

Identification of new compounds

In this study, four metabolic pathways were constructed based on the secondary metabolism of flavonols. Pathway 1 is a pathway for glycosylation and methylation at the C-3, C-7, C-4' sites with desmethylicaritin as the parent nucleus (Fig. 1). In addition, 41 components in this pathway have been recorded in the database, and two new compounds (M1, M2) were identified by HRMS.

Pathway 2 is a glycosylation and methylation pathway at the C-3, C-7, and C-4' sites with the hydration product of the isoprenyl double bond at the C-8 site of desmethylicaritin (Fig. 2). This pathway involves a total of 21 compounds, four of which are known components (noricarliin, maohuoside A, icaritin-3-O-α-rhamnoside, and wanepimedoside A). In addition, one new compound (M3) and one new component (M4) have been identified by HRMS.

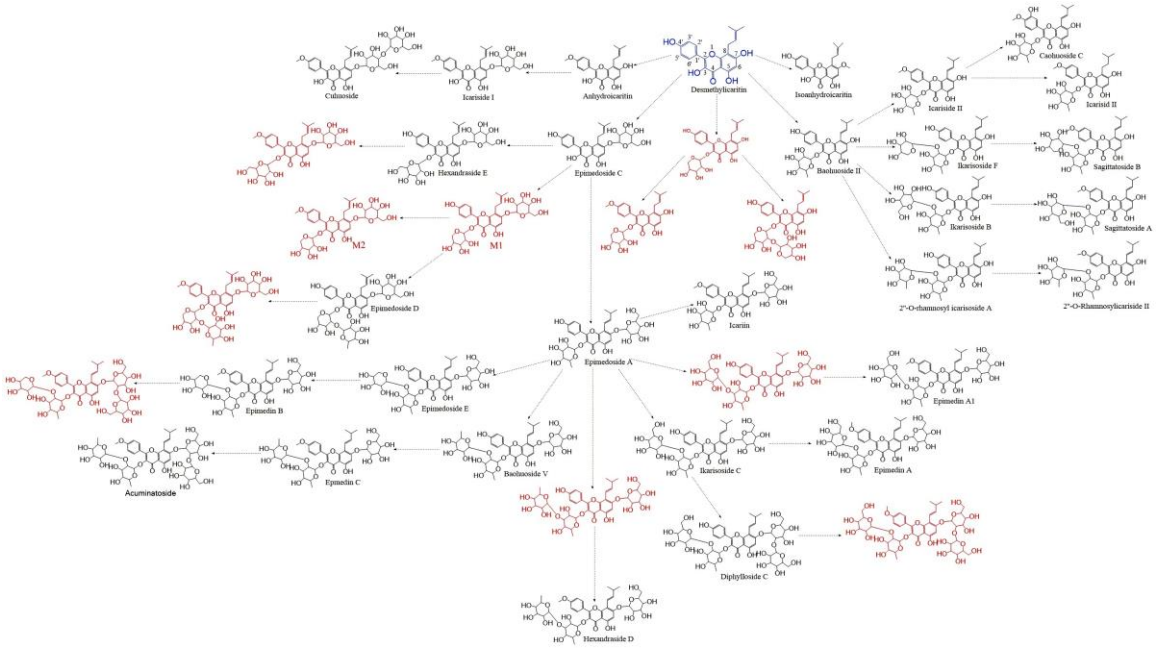

Fig. 1: Secondary metabolic network of pathway 1.

Note: Molecular formulae in blue were the parent nuclei, in black were the known components and in red were the derived compounds.

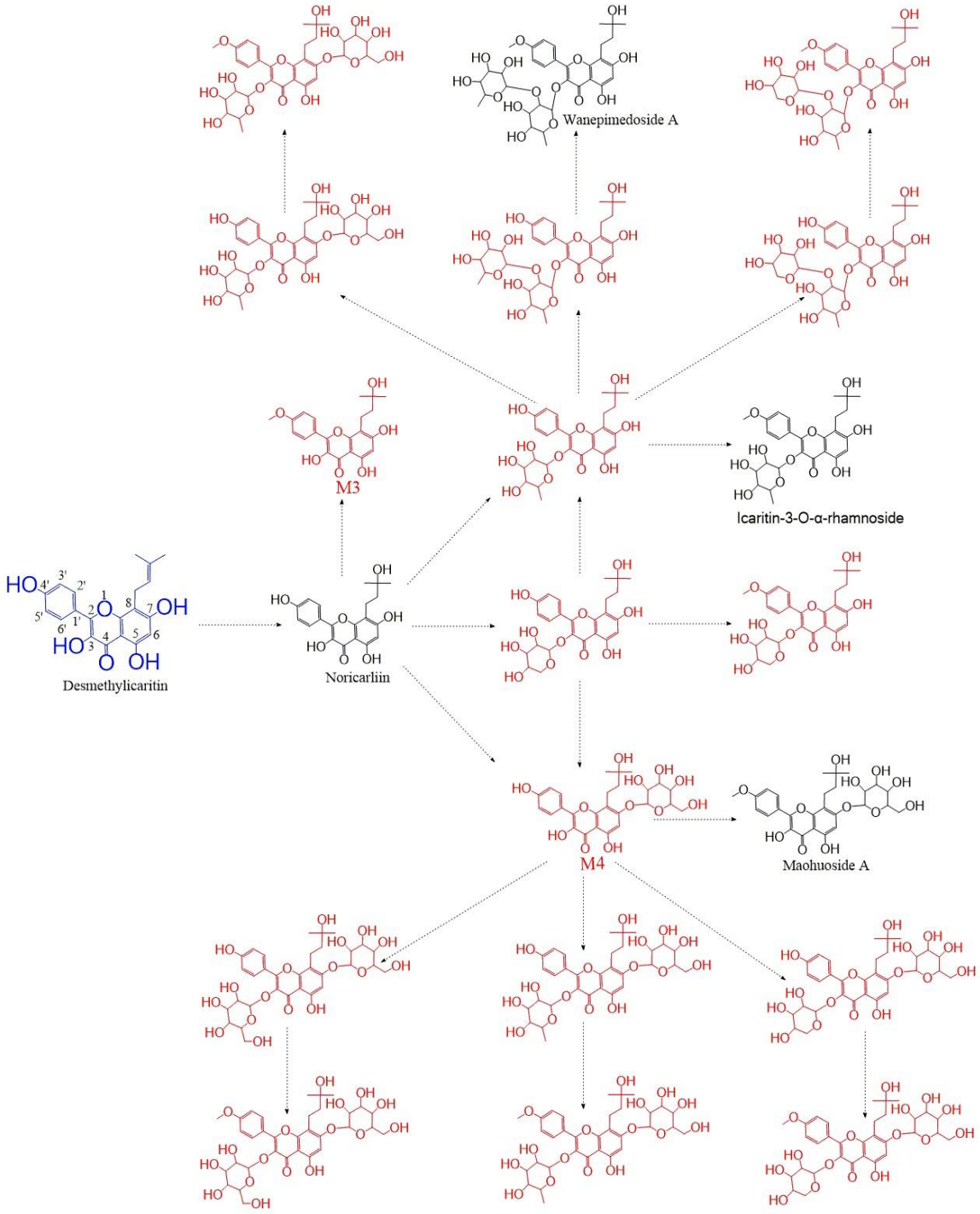

Fig. 2: Secondary metabolic network of pathway 2.

Note: Molecular formulae in blue were the parent nuclei, in black were the known component and in red were the derived compound.

Pathway 3 is the glycosylation and methylation pathway at the C-3, C-7, and C-4' sites with the hydrogenated product of the isoprenyl double bond at the C-8 site of desmethylicaritin (Fig. 3). The pathway involves 21 compounds, of which icarisid II was the only known component. Four new compounds (M5-M8) were identified by HRMS.

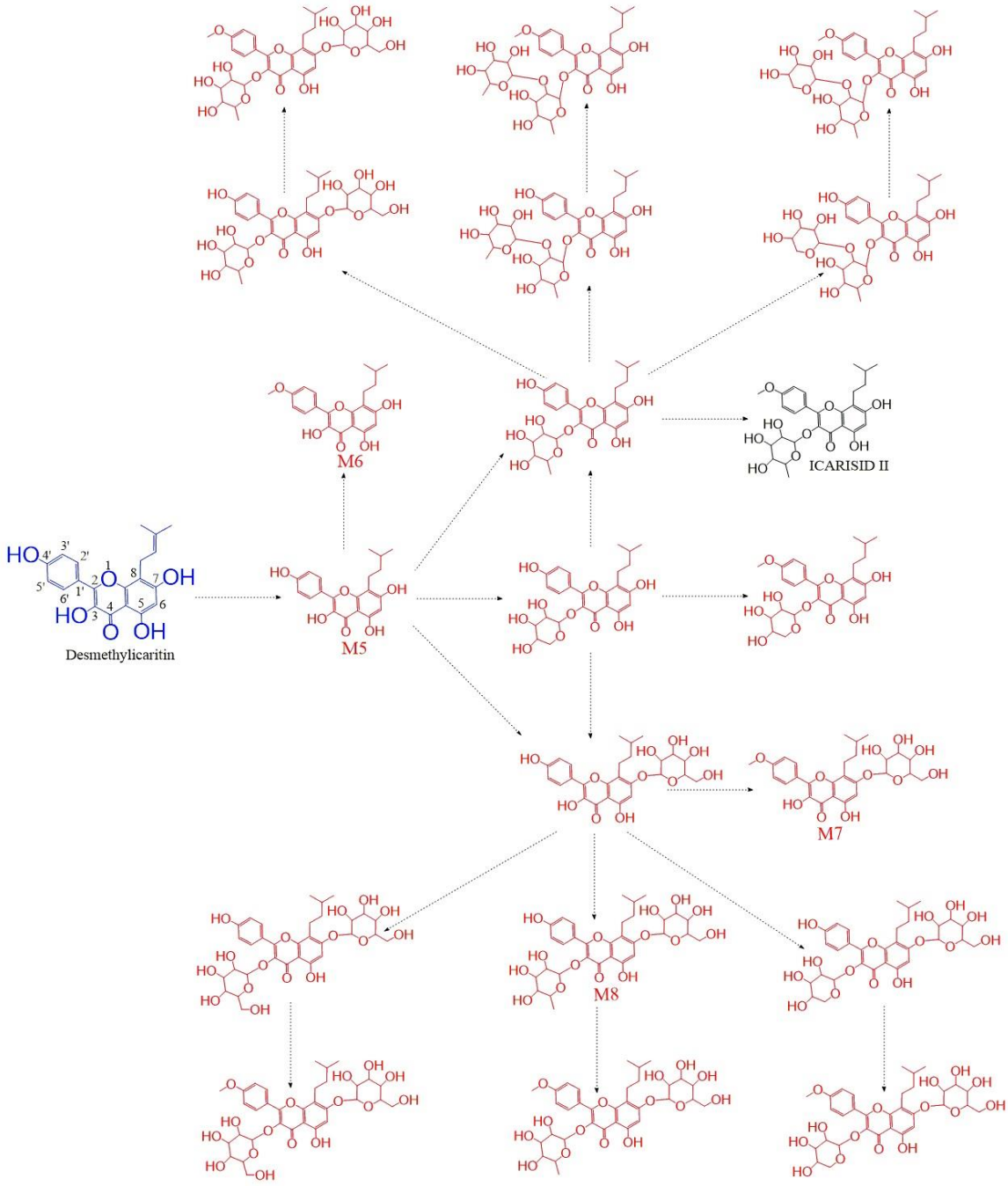

Fig. 3: Secondary metabolic network of pathway 3.

Note: Molecular formulae in blue were the parent nuclei, in black were the known component and in red were the derived compound.

Pathway 4 is the glycosylation and methylation pathway at the C-3 and C-4' sites that takes the addition product of the C-7 and C-8 groups of desmethylicaritin as the parent nucleus (Fig. 4). A total of 22 compounds were involved in this pathway, of which 3 are known components (anhydrocicaritin, ikarisoside E, and acuminatin), and 6 are new compounds (M9，M11-M15) and 1 new component (M10). The identification results are shown in Table 2. The total ion flow diagram of the samples is shown in Fig. 5.

Verification and analysis of new components by HRMS

The molecular formula of the new compound M1 is $C_{31}H_{36}O_{15}$. The theoretical value and the measured value deviation of $[M+H]^+$ is 0.7 ppm. According to the fragmentation diagram of secondary fragments (Fig. 6), the precursor ion of *m/z* 649.2237 ($[M+H]^+$) continuous removal of 132.0446 Da, 162.0565 Da, and 56.0636 Da groups generated fragment ions of *m/z* 517.1791, *m/z* 355.1226, and *m/z* 299.0590. According to the Mass Calculators function of PeakView software, the accurate mass of $C_5H_8O_4$, $C_6H_{10}O_5$, and C_4H_8 is 132.0417 Da, 162.0523 Da, and 56.0621 Da, respectively. New debris difference is < 0.01 Da. This compound contains xylosides, glucosides, and prenyl structures, which is consistent with the results of metabolic pathway deduction. After the compounds' information was imported into SciFinder, Reaxys, and ChemSpider for searching, no chemical structure matching M1 was found; thus, it was speculated that M1 is a new compound of *Epimedium*

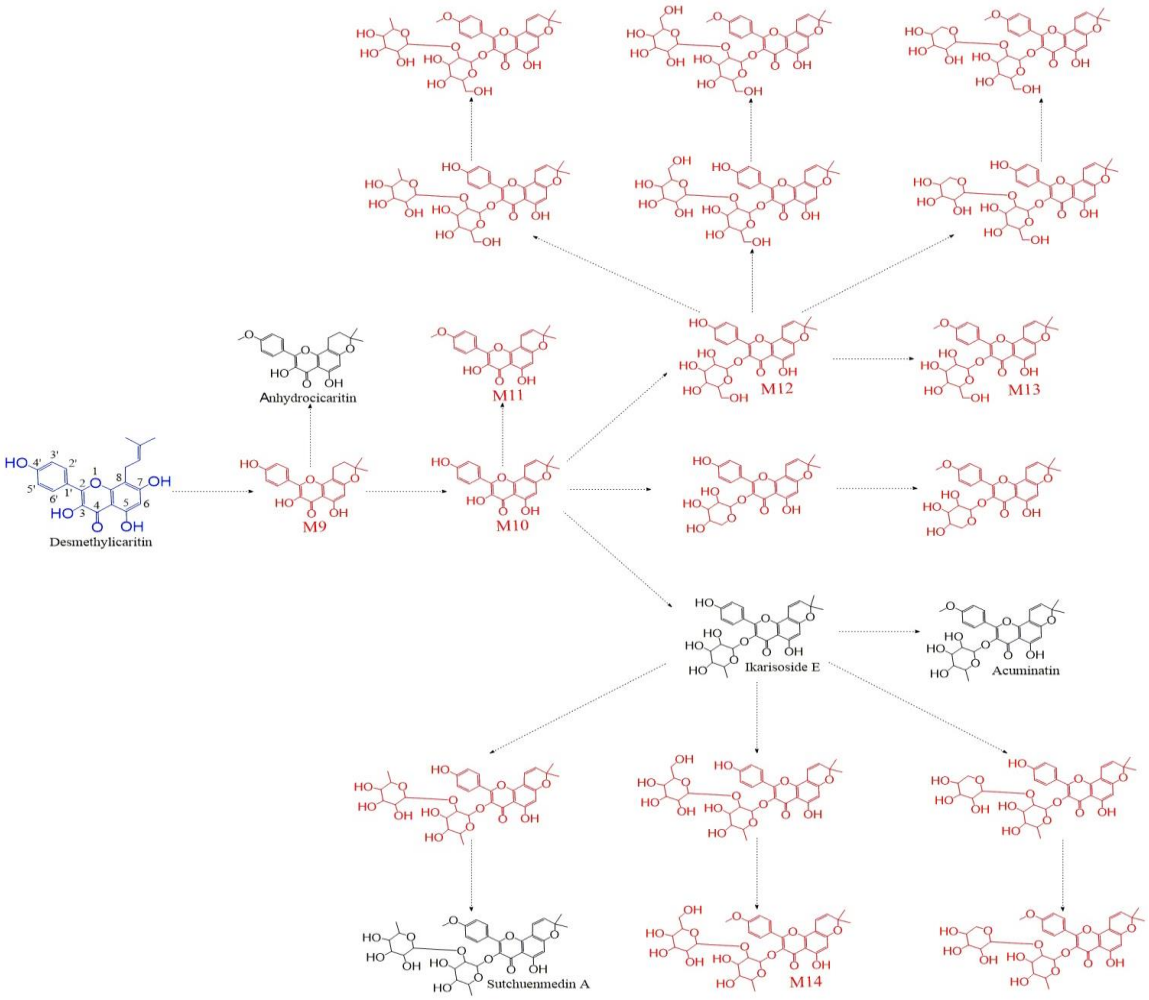

Fig. 4: Secondary metabolic network of pathway **4**.

Note: Molecular formulae in blue were the parent nuclei, in black were the known component and in red were the derived compound.

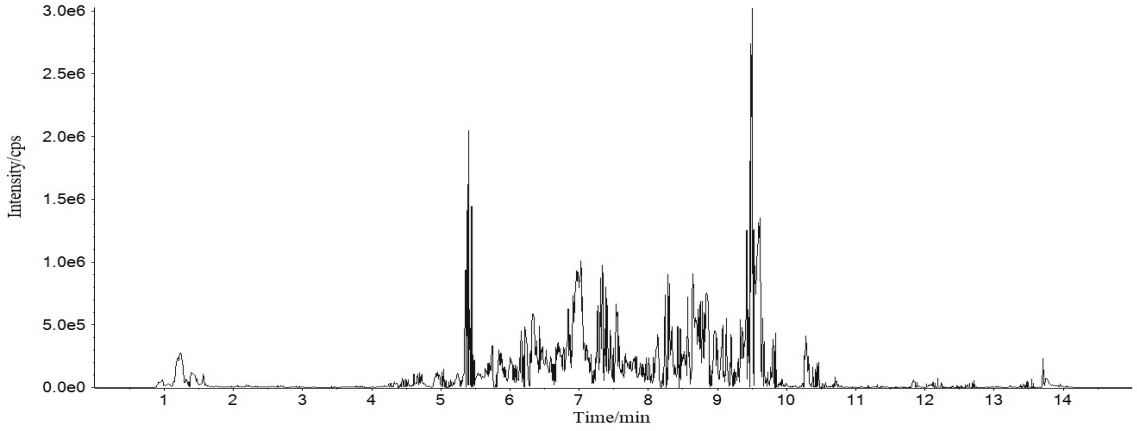

Fig. 5: Total ion chromatogram of the sample.

Table-2: Identification results of new compounds in *Epimedium.*

Compound No.	RT (min)	Ion mode	Mass charge ratio(*m/z*)			Deviation (ppm)	Formula	Intensity (cps)	Identification results
			Calculated mass	Measured mass	secondary fragment				
M1	7.58	$[M+H]^+$	649.2127	649.2132	517.1791, 355.1226, 299.0590	0.7	$C_{31}H_{36}O_{15}$	25460	New compound
M2	7.08	$[M+H]^+$	663.2284	663.2308	501.1821, 369.1374, 355.1211	3.7	$C_{32}H_{38}O_{15}$	18861	New compound
M3	7.75	$[M+H]^+$	387.1438	387.1441	369.1361, 313.0731	0.6	$C_{21}H_{22}O_7$	24357	New compound
M4	7.65	$[M+H]^+$	535.1810	535.1801	373.1306, 355.1199, 337.1082	1.6	$C_{26}H_{30}O_{12}$	156727	Amurensin
M5	8.76	$[M+H]^+$	357.1333	357.1346	301.0640	3.8	$C_{20}H_{20}O_6$	50425	New compound
M6	9.63	$[M+H]^+$	371.1489	371.1486	313.0643, 301.0638	-0.9	$C_{21}H_{22}O_6$	209947	New compound
M7	7.37	$[M+H]^+$	533.2017	533.1996	371.1438, 353.1058, 315.0792	-3.9	$C_{27}H_{32}O_{11}$	305985	New compound
M8	7.10	$[M+H]^+$	665.2440	665.2426	519.1815, 357.1271, 301.0633	-2.1	$C_{32}H_{40}O_{15}$	33395	New compound
M9	8.29	$[M+H]^+$	355.1176	355.1193	337.1105, 319.1001, 307.0634	4.6	$C_{20}H_{18}O_6$	160631	New compound
M10	8.61	$[M+H]^+$	353.1020	353.1034	335.0934, 295.0627	4.2	$C_{20}H_{16}O_6$	310345	Citrusinol
M11	9.29	$[M+H]^+$	367.1176	367.1194	352.0987, 309.0797, 294.0564	4.8	$C_{21}H_{18}O_6$	131786	New compound
M12	7.76	$[M+H]^+$	515.1548	515.1556	353.1047, 335.0940	1.6	$C_{26}H_{26}O_{11}$	7290	New compound
M13	7.78	$[M+H]^+$	529.1704	529.1727	367.1204, 349.1084	4.2	$C_{27}H_{28}O_{11}$	5943	New compound
M14	6.99	$[M+H]^+$	675.2284	675.2309	513.1804, 367.1208, 325.0729	3.8	$C_{33}H_{38}O_{15}$	21335	New compound

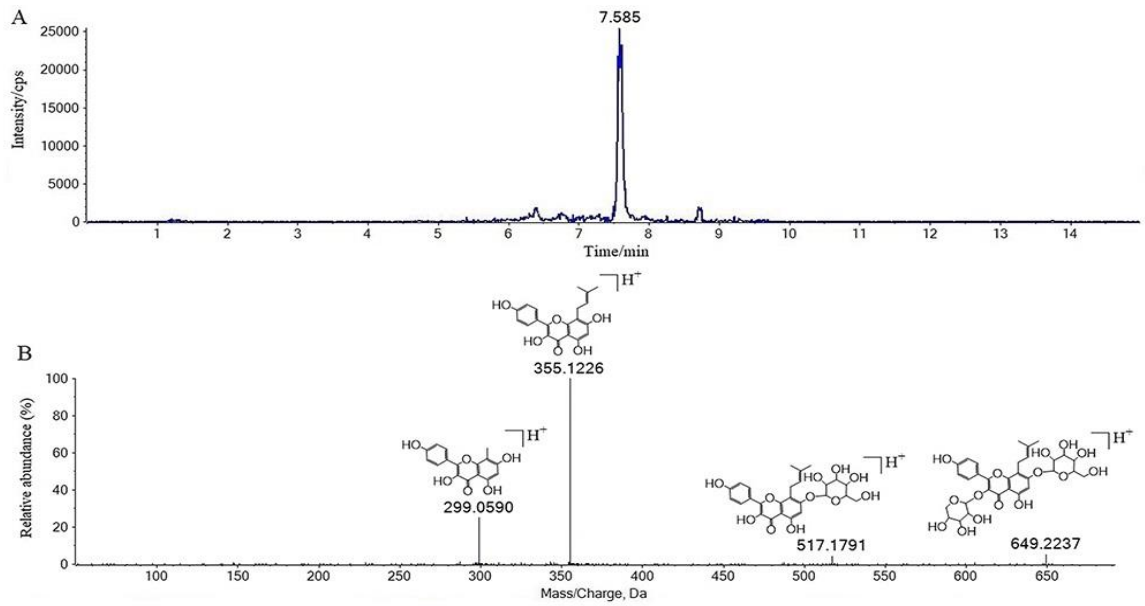

Fig. 6: Extraction ion diagram (A) and second-order fragmentation rule diagram (B) of compound M1.

The molecular formula of the new compound M2 is $C_{32}H_{38}O_{15}$. The theoretical value and the measured value deviation of $[M+H]^+$ is 3.7 ppm. According to the fragmentation diagram of secondary fragments (Fig. 7), the precursor ion of *m/z* 663.2398 ($[M+H]^+$) continuous removal of 162.0577 Da, 132.0447 Da and 14.0163 Da groups generated fragment ions of *m/z* 501.1821, *m/z* 369.1374 and *m/z* 355.1211. The accurate mass of $C_6H_{10}O_5$, $C_5H_8O_4$, and CH_2 is 162.0529 Da, 132.0417 Da, and 14.0151 Da, respectively. The compound contains glucoside, xylosid, and methyl structures, which is consistent with the results of metabolic pathway deduction. After the compound information was imported into SciFinder, Reaxys, and ChemSpider, no chemical structure matching M2 was found; thus, it was speculated that M2 is a new compound of *Epimedium.*

The molecular formula of the new compound M3 is $C_{21}H_{22}O_7$. The theoretical value and the measured value deviation is 0.6 ppm. According to Fig. 8, the precursor ion of *m/z* 387.1509 ($[M+H]^+$) continuous removal of 18.0148 Da, 56.0630 Da groups generated fragment ions of *m/z* 369.1361 and *m/z* 313.0731. The exact mass of H_2O and C_4H_8 is 18.0100 Da and 56.0621 Da when using PeakView software. All the removal debris difference is < 0.01 Da. The compound contains glucoside, xylosid and methyl structures, which was consistent with the results of metabolic pathway deduction. After the compound information was imported into SciFinder, Reaxys, ChemSpider, and other online databases, no chemical structure matching M3 were found; thus, it was speculated that M3 is a new compound of *Epimedium.*

The molecular formula of the new compound M4 is $C_{26}H_{30}O_{12}$. The theoretical value and the measured value deviation of $[M+H]^+$ is 1.6 ppm. According to the fragmentation diagram of secondary fragments (Fig. 9), the precursor ion of *m/z* 535.1862 ($[M+H]^+$) continuous removal of 162.0556 Da, 18.0107 Da and 18.0117 Da groups generated fragment ions of *m/z* 373.1306, *m/z* 355.1199 and *m/z* 337.1082. The exact mass of $C_6H_{10}O_5$ and H_2O is 162.05228 Da and 18.01002 Da, respectively, calculated by PeakView software. The removal debris difference is < 0.01 Da. The compound contains glucoside, xylosid, and methyl structures, which were consistent with the results of metabolic pathway deduction. After the compound information was imported into SciFinder, Reaxys, ChemSpider, and other online databases, only the chemical structure of amurensin was found to match M4; thus, it was speculated that amurensin is a new component of *Epimedium.*

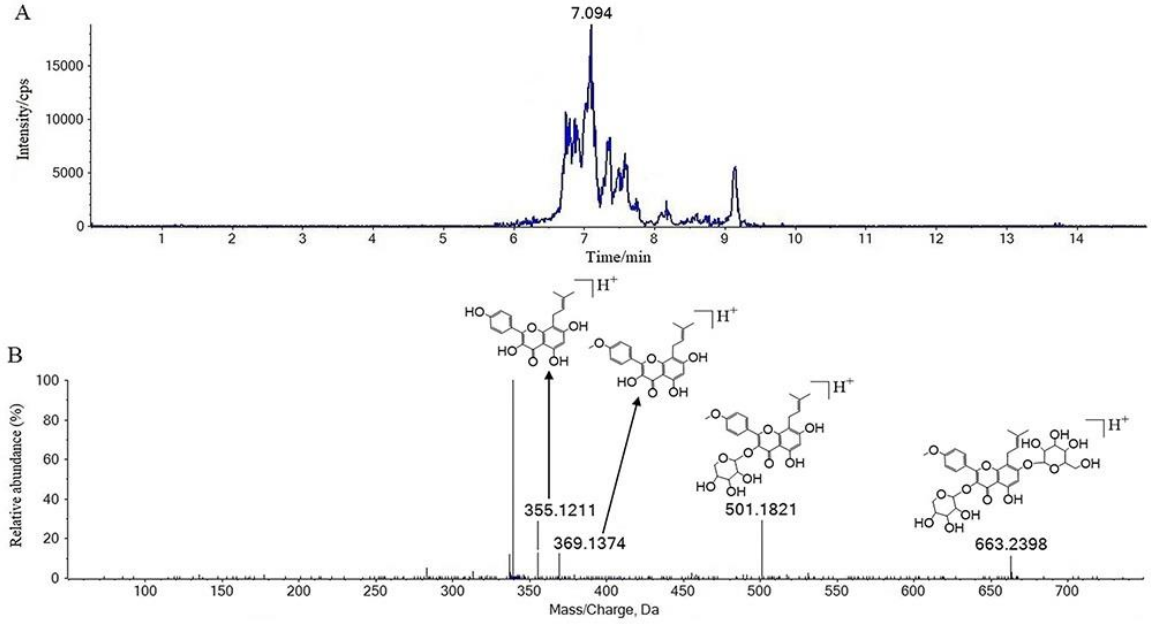

Fig. 7: Extraction ion diagram (A) and second-order fragmentation rule diagram (B) of compound M2.

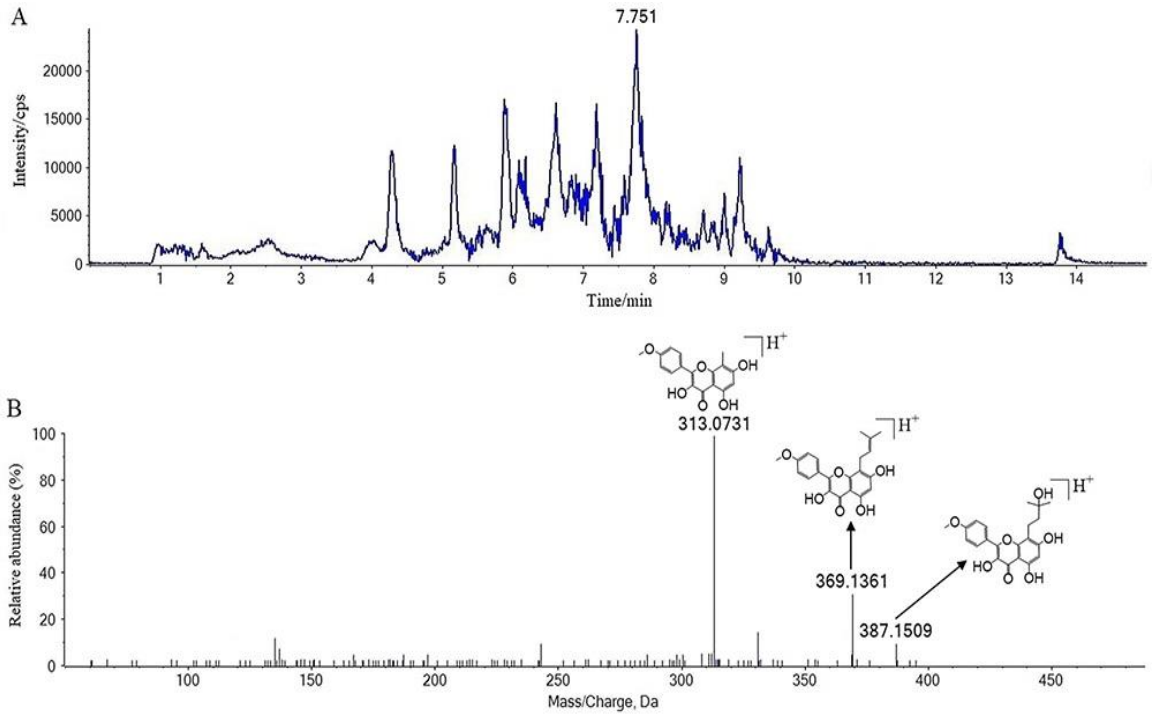

Fig. 8: Extraction ion diagram (A) and second-order fragmentation rule diagram (B) of compound M3.

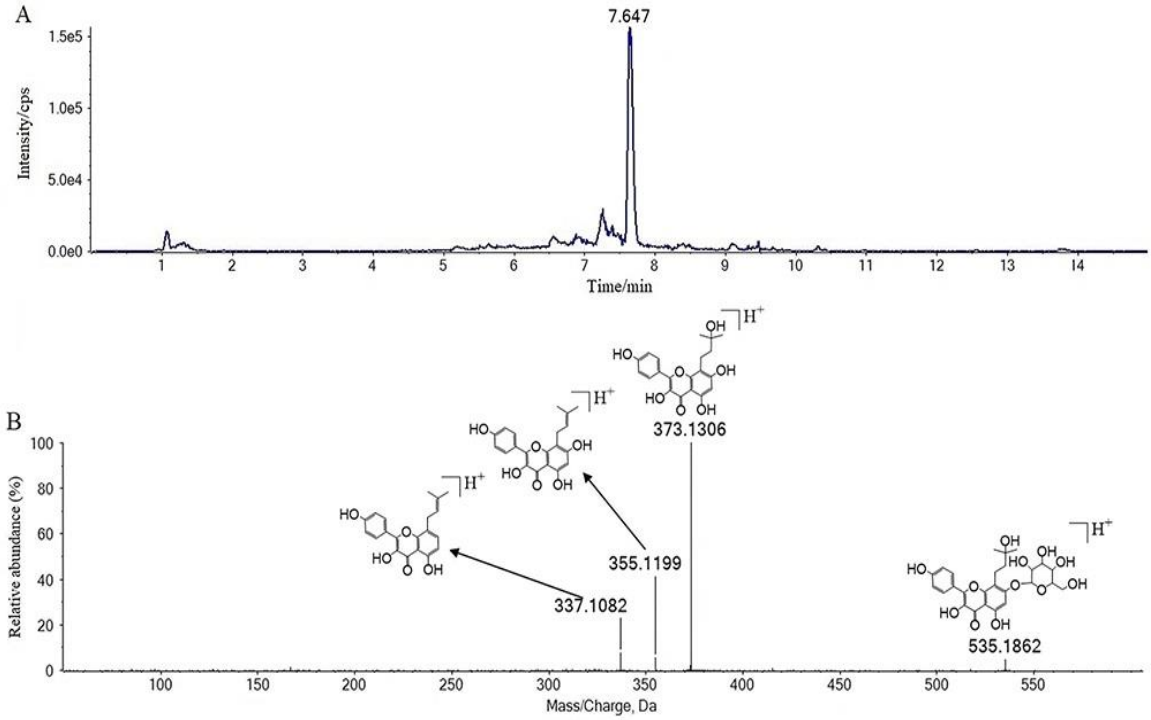

Fig. 9: Extraction ion diagram (A) and second-order fragmentation rule diagram (B) of compound M4.

The molecular formula of the new compound M5 is $C_{20}H_{20}O_6$. The theoretical value and the measured value deviation is 3.8 ppm. According to the fragmentation diagram of secondary fragments (Fig. 10), the precursor ion of *m/z* 357.1292 ($[M+H]^+$) removal of 56.0652 Da groups generated fragment ion of *m/z* 301.0640. The exact mass of C_4H_8 is 56.06205 Da calculated by PeakView software. The removal debris difference was less than 0.01 Da. The compound contains isopentyl structure, which was consistent with the results of metabolic pathway deduction. After the compound information was imported into SciFinder, Reaxys, ChemSpider, and other online databases for searching, no chemical structure matching M5 were found; thus, it was speculated that M5 is a new compound of *Epimedium*.

The molecular formula of the new compound M6 is $C_{21}H_{22}O_6$. The theoretical value and the measured value deviation was 0.9 ppm. According to the fragmentation diagram of secondary fragments (Fig. 11), the precursor ion of *m/z* 371.1441 ($[M+H]^+$) continuous removal of 58.0798 Da and 70.0803 Da groups generated fragment ions of *m/z* 313.0643 and *m/z* 301.0638. The exact mass of C_4H_{10} and C_5H_{10} is 58.0777 Da and 70.0777 Da, respectively, calculated by PeakView software. The removal debris difference is less than 0.01 Da. The compound contains isopentyl structure, which was consistent with the results of metabolic pathway deduction. After the compound information was imported into SciFinder, Reaxys, ChemSpider, and other online databases for searching, no chemical structure matching M6 were found; thus, it was speculated that M6 is a new compound of *Epimedium*.

The molecular formula of the new compound M7 is $C_{27}H_{32}O_{11}$. The theoretical value and the measured value deviation was 3.9 ppm. According to the fragmentation diagram of secondary fragments (Fig. 12), the precursor ion of *m/z* 533.1700 ($[M+H]^+$) continuous removal of 162.0262 Da and 180.0642 Da groups generated fragment ions of *m/z* 371.1438 and *m/z* 353.1058; the removal of 56.0646 Da ion by *m/z* of 371.1438 generate a fragment ion of m/z 315.0792. The exact mass of $C_6H_{10}O_5$, $C_6H_{12}O_6$, and C_4H_8 is 162.0523 Da, 180.0628 Da, and 56.0621 Da, respectively, calculated by PeakView software. The removal debris difference is less than 0.01 Da except for the 162 group. The compound contains xylosides, glucosides, and prenyl structures, which was consistent with the results of metabolic pathway deduction. After the compound information was imported into SciFinder, Reaxys, ChemSpider, and other online databases, no chemical structure matching M7 were found; thus, it was speculated that M7 is a new compound of *Epimedium*.

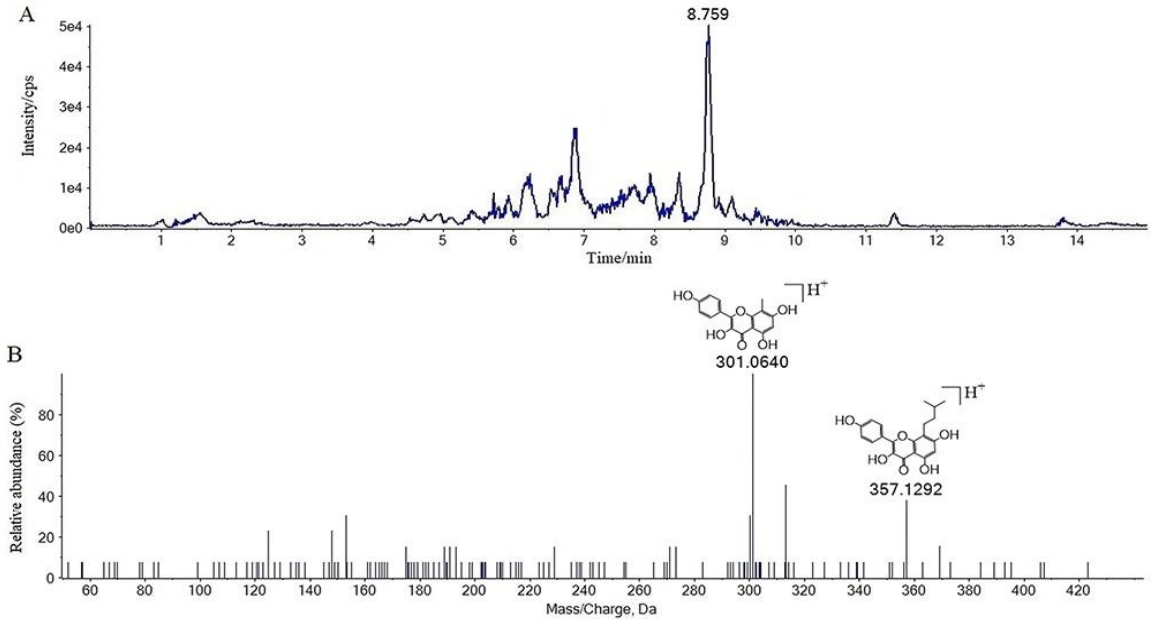

Fig. 10: Extraction ion diagram (A) and second-order fragmentation rule diagram (B) of compound M5.

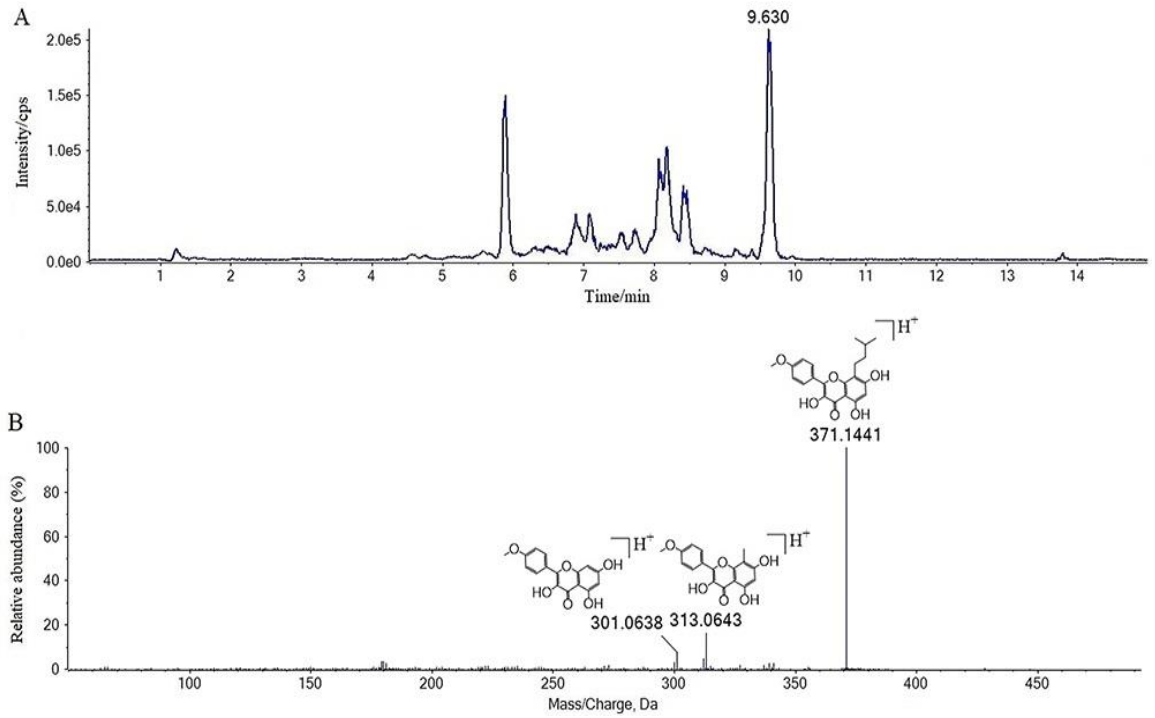

Fig. 11: Extraction ion diagram (A) and second-order fragmentation rule diagram (B) of compound M6.

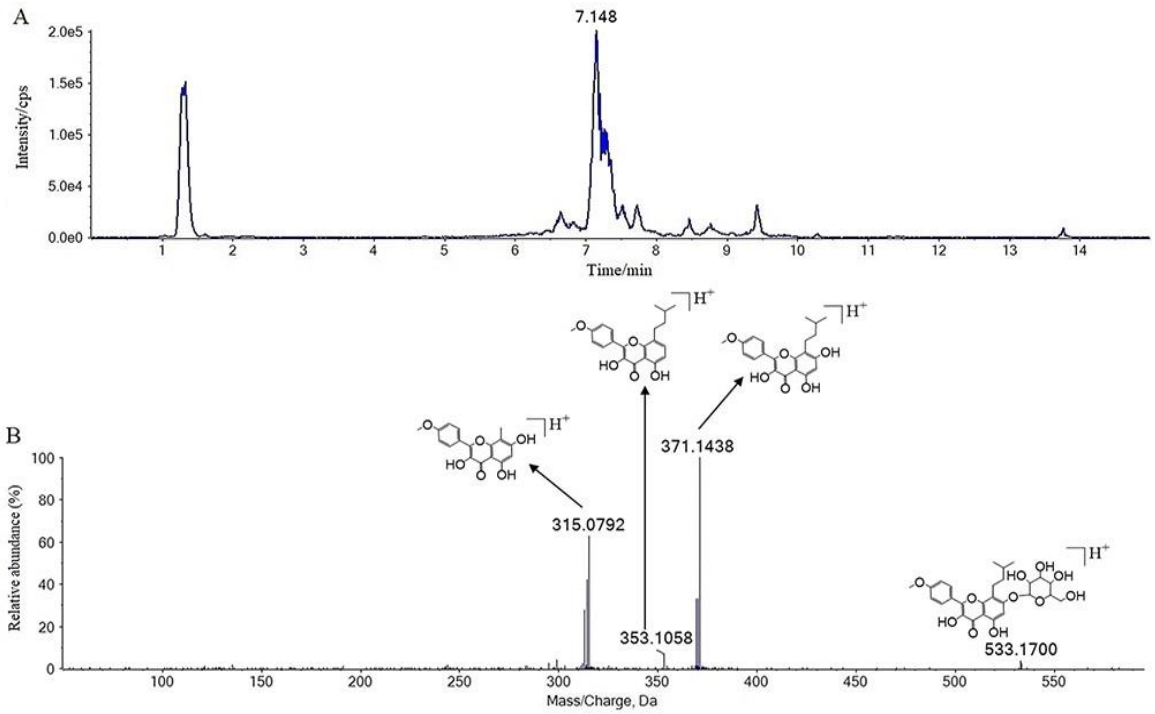

Fig. 12: Extraction ion diagram (A) and second-order fragmentation rule diagram (B) of compound M7.

The molecular formula of the new compound M8 is $C_{32}H_{40}O_{15}$. The theoretical value and the measured value deviation was 2.1 ppm. According to the fragmentation diagram of secondary fragments (Fig. 13), the precursor ion of *m/z* 665.3763 ($[M+H]^+$) continuous removal of 146.1948 Da, 162.0544 Da and 56.0638 Da groups generated fragment ions of *m/z* 519.1815, *m/z* 357.1271 and *m/z* 301.0633. The exact mass of $C_6H_{10}O_4$, $C_6H_{10}O_5$ and C_4H_8 is 146.0574 Da, 162.0523 Da, and 56.0621 Da, respectively, calculated by PeakView software. The removal debris difference is less than 0.01 Da, except for the 146 group. The compound contains the structure of rhamnoside, glucoside, and isopentyl, which was consistent with the results of metabolic pathway deduction. After the compound information was imported into SciFinder, Reaxys, ChemSpider, and other online databases for searching, no chemical structure matching M8 were found. Thus, it is speculated that M8 is a new compound of *Epimedium*.

The molecular formula of the new compound M9 is $C_{20}H_{18}O_6$. The theoretical value and the measured value deviation is 4.6 ppm. According to the fragmentation diagram of secondary fragments (Fig. 14), the precursor ion of *m/z* 355.1206 ($[M+H]^+$) continuous removal of 18.0104 Da, 30.0471 Da and 54.0476 Da groups generated fragment ions of *m/z* 319.1001, *m/z* 307.0634 and *m/z* 283.0629. The exact mass of H_2O, $C_9H_{14}O_2$, $C_{12}H_{10}O_5$, C_2H_6, and C_4H_6 is 18.0100 Da, 154.0988 Da, 234.0523 Da, 30.0464 Da, and 54.0464 Da, respectively, calculated by PeakView software. All the removal debris difference was less than 0.01 Da. The compound contains hydroxyl and prenyl structures, which was consistent with the results of metabolic pathway deduction. After the compound information was imported into SciFinder, Reaxys, ChemSpider, and other online databases, no chemical structure matching M9 were found; thus, it was speculated that M9 is a new compound of *Epimedium*.

The molecular formula of the new compound M10 is $C_{20}H_{16}O_6$, with a deviation of 4.2 ppm. According to the fragmentation diagram of secondary fragments (Fig. 15), the precursor ion of *m/z* 353.1056 ($[M+H]^+$) continuous removal of 18.0122 Da and 40.0307 Da groups generated fragment ions of *m/z* 335.0934 and *m/z* 295.0627. The exact mass of H_2O and C_3H_4 is 18.0100 Da and 40.0308 Da, respectively, calculated by PeakView software. The removal debris difference was less than 0.01 Da. The compound contains hydroxyl and hydrocarbon groups, which were consistent with the results of metabolic pathway deduction. After the compound information was imported into SciFinder, Reaxys, ChemSpider, and other online databases for searching, the chemical structure of citrusinol was found to match M10. Thus, it was speculated that citrusinol is a new component of *Epimedium*.

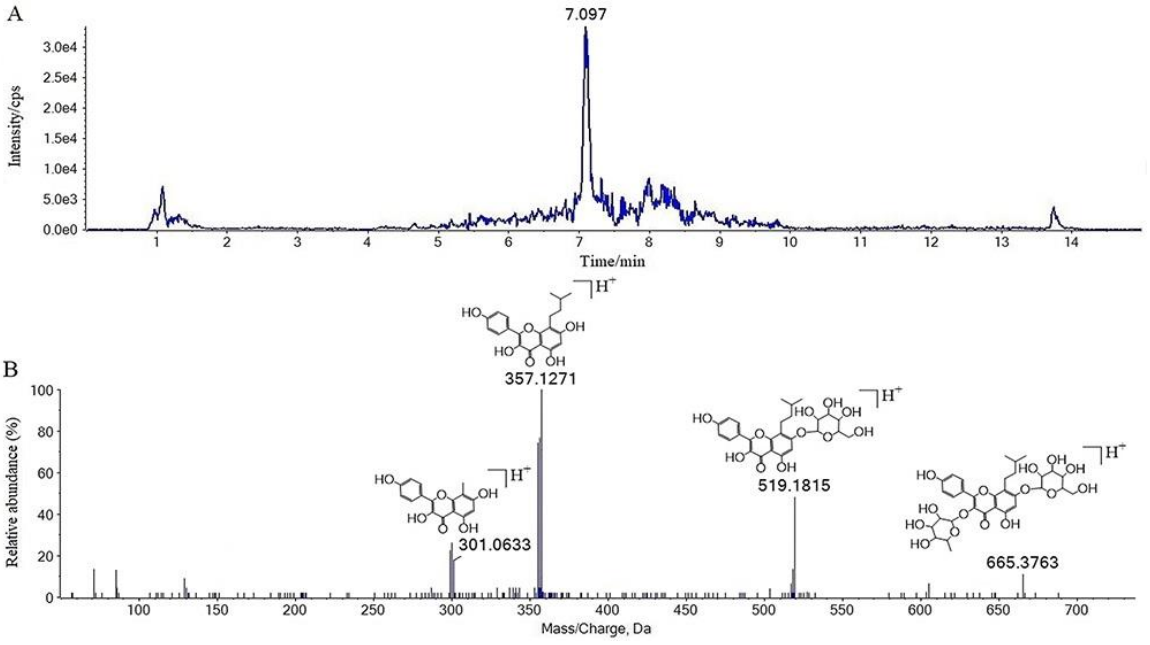

Fig. 13: Extraction ion diagram (A) and second-order fragmentation rule diagram (B) of compound M8.

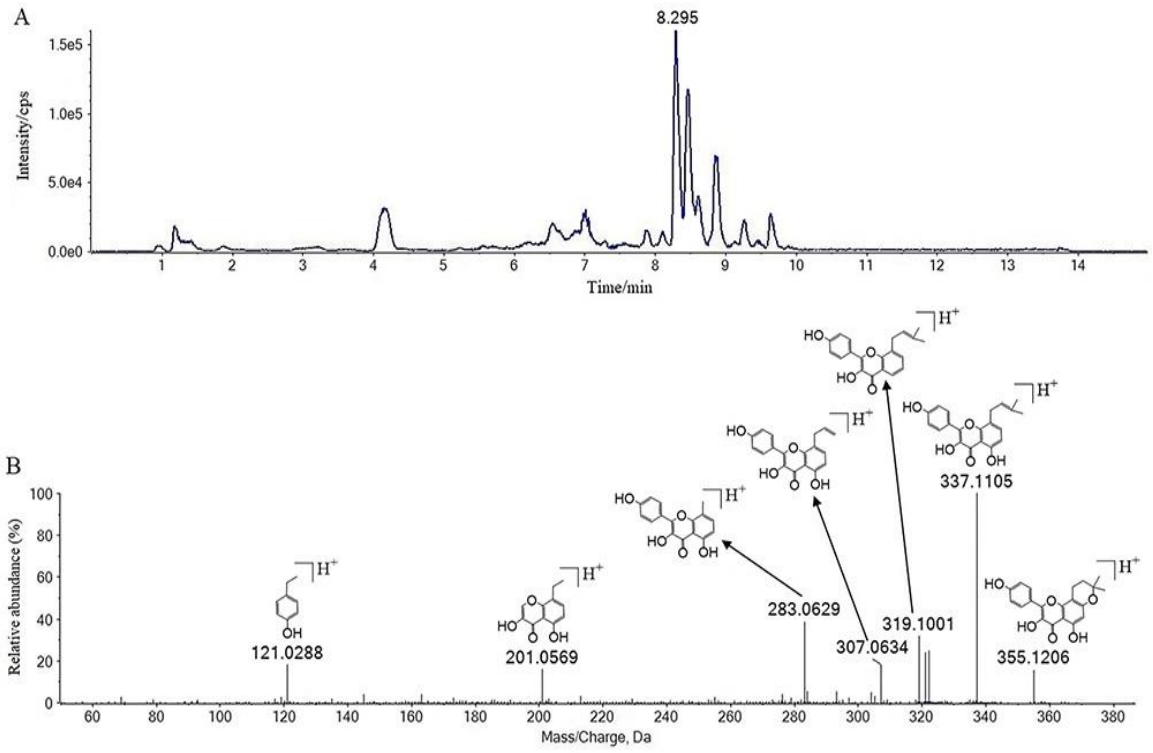

Fig. 14: Extraction ion diagram (A) and second-order fragmentation rule diagram (B) of compound M9.

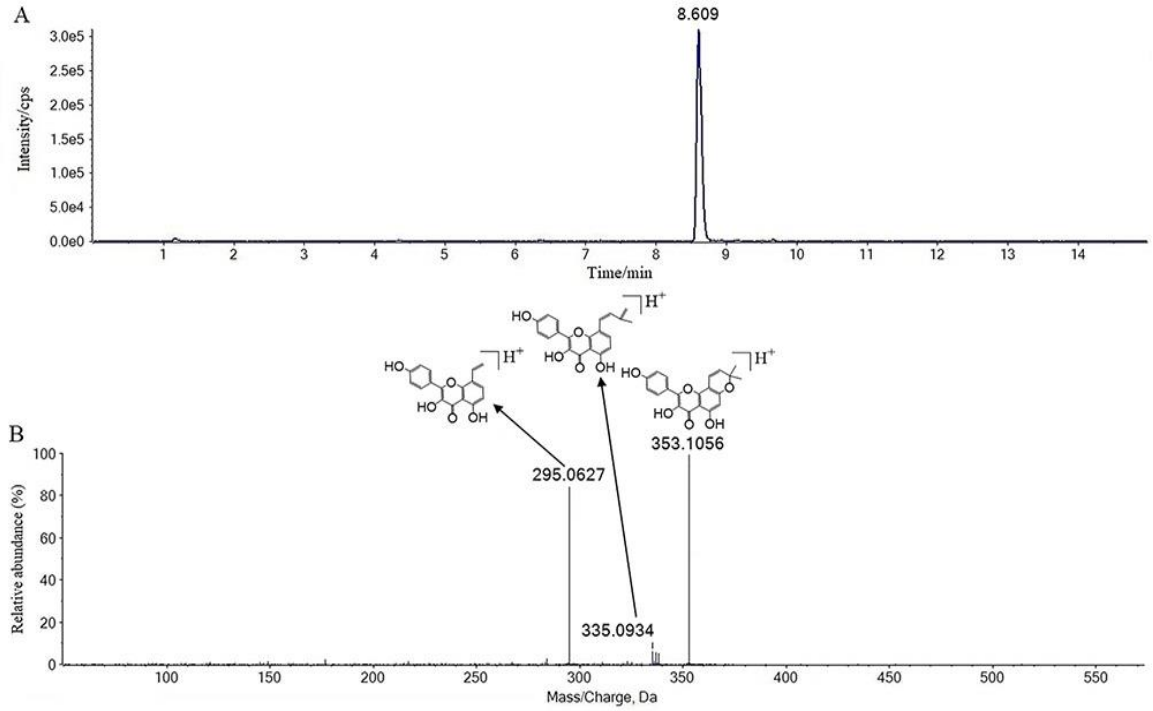

Fig. 15: Extraction ion diagram (A) and second-order fragmentation rule diagram (B) of compound M10.

The molecular formula of the new compound M11 is $C_{21}H_{18}O_6$, with a deviation of 4.8 ppm. According to the fragmentation diagram of secondary fragments (Fig. 16), the precursor ion of *m/z* 367.1227 ($[M+H]^+$) continuous removal of 15.0240 Da and 58.0430 Da groups generated fragment ions of *m/z* 352.0987 and *m/z* 309.0797. The exact mass of CH_3, C_3H_6O, and CO is 15.0229 Da, 58.0413 Da, and 27.9944 Da, respectively calculated by PeakView software. All the removal debris difference was less than 0.01 Da. The compound contains methyl, alkoxy, and carbonyl structures, which was consistent with the results derived from metabolic pathways. After the compound information was imported into SciFinder, Reaxys, ChemSpider, and other online databases, no chemical structure matching M11 were found; thus, it was speculated that M11 is a new compound of *Epimedium.*

The molecular formula of the new compound M12 is $C_{26}H_{26}O_{11}$, with a deviation of 1.6 ppm. According to the fragmentation diagram of secondary fragments (Fig. 17), the precursor ion of *m/z* 515.1619 ($[M+H]^+$) continuous removal of 162.0572 Da and 18.0107 Da groups generated fragment ions of *m/z* 353.1047 and *m/z* 335.0940. The exact mass of $C_6H_{10}O_5$ and H_2O is 162.0523 Da and 18.0100 Da, respectively, calculated by PeakView software. All the removal debris difference was less than 0.01 Da. The compound contains glucoside and hydroxyl structures, which was consistent with the results of metabolic pathway deduction. After the compound information was imported into SciFinder, Reaxys, ChemSpider, and other online databases, no chemical structure matching M12 were found; thus, it was speculated that M12 is a new compound of *Epimedium*.

The molecular formula of the new compound M13 is $C_{27}H_{28}O_{11}$, with a deviation of 4.2 ppm. According to the fragmentation diagram of secondary fragments (Fig. 18), the precursor ion of *m/z* 529.3371 ($[M+H]^+$) continuous removal of 162.2167 Da and 18.0120 Da groups generated fragment ions of *m/z* 367.1204 and *m/z* 349.1084. The exact mass of $C_6H_{10}O_5$ and H_2O is 162.0523 Da and 18.0100 Da, respectively, calculated by PeakView software, while the difference of debris removal was 0.164 Da and 0.002 Da. The compound contains glucoside and hydroxyl structures, which was consistent with the results of metabolic pathway deduction. After the compound information was imported into SciFinder, Reaxys, ChemSpider, and other online databases, no chemical structure matching M13 was found; thus, it was speculated that M13 is a new compound of *Epimedium*.

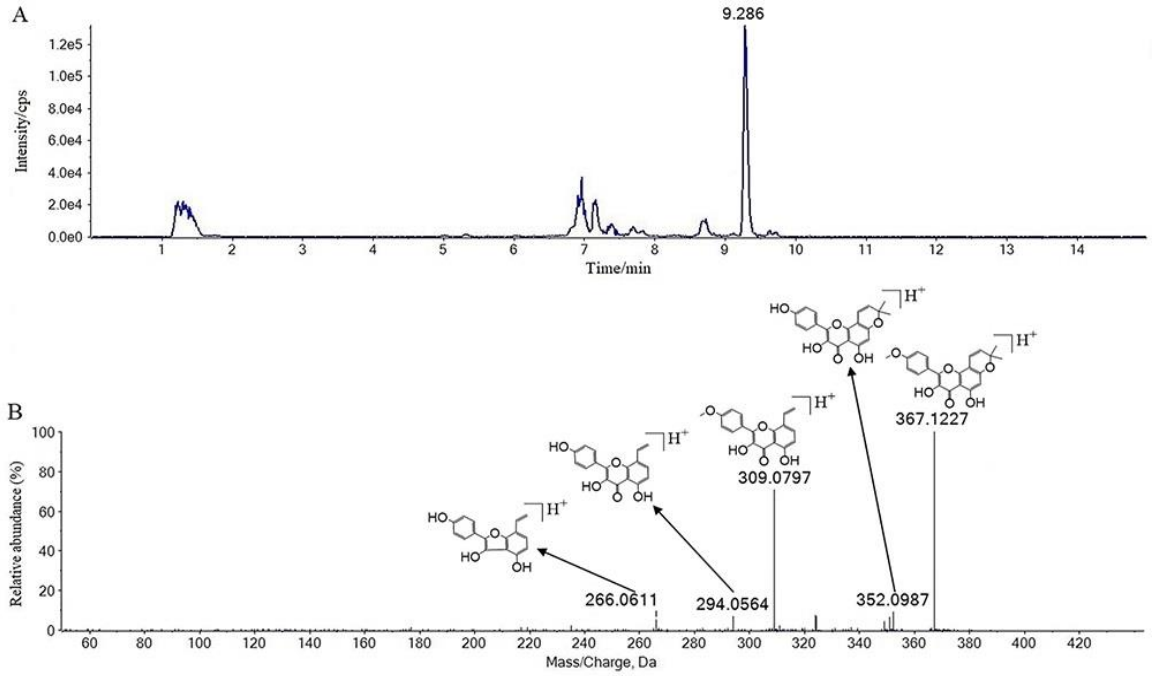

Fig. 16: Extraction ion diagram (A) and second-order fragmentation rule diagram (B) of compound M11.

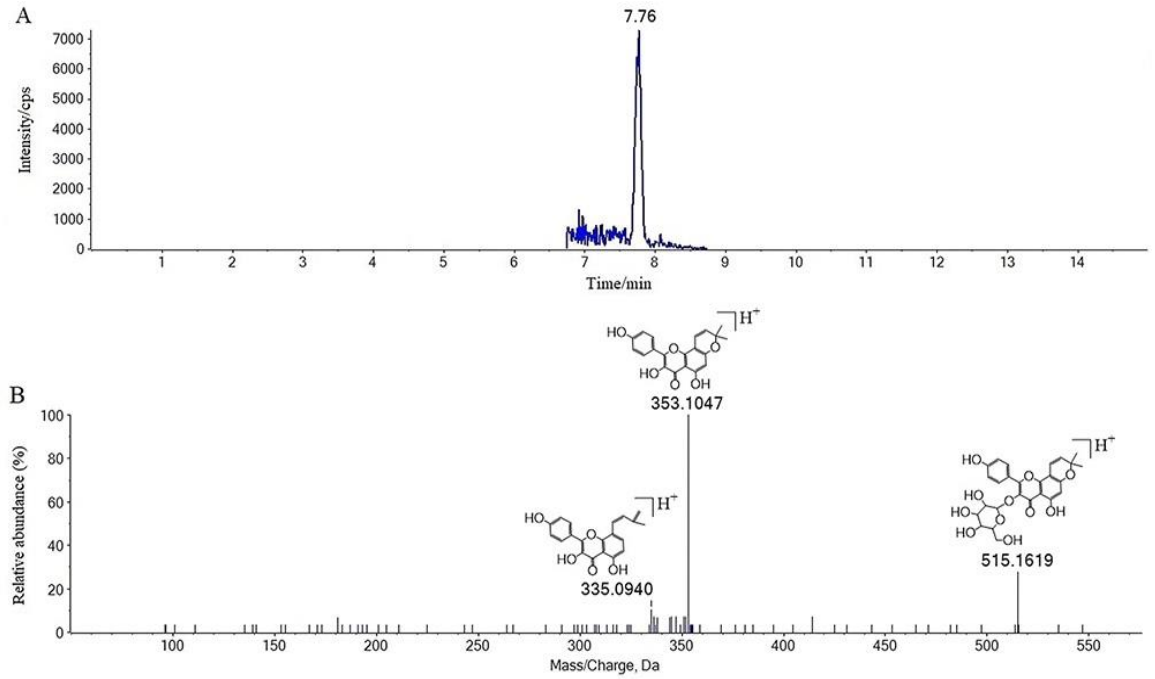

Fig. 17: Extraction ion diagram (A) and second-order fragmentation rule diagram (B) of compound M12.

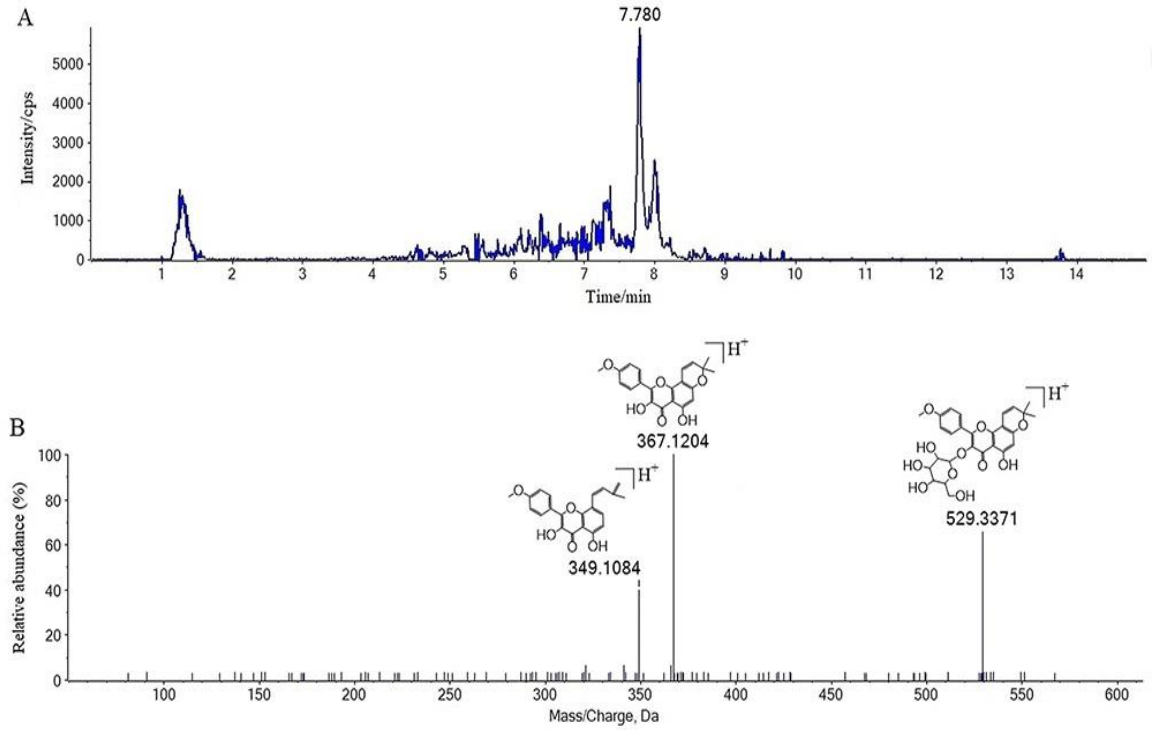

Fig. 18: Extraction ion diagram (A) and second-order fragmentation rule diagram (B) of compound M13.

The molecular formula of the new compound M14 is $C_{33}H_{38}O_{15}$, with a deviation of 3.8 ppm. According to the fragmentation diagram of secondary fragments (Fig. 19), the precursor ion of *m/z* 675.2292 ($[M+H]^+$) continuous removal of 162.0488 Da, 146.0596 Da and 42.0479 Da groups generated fragment ions of *m/z* 367.1208 and *m/z* 325.0729. The exact mass of $C_6H_{10}O_5$, $C_6H_{10}O_4$, and C_3H_6 is 162.05228 Da, 146.0574 Da, and 42.0464 Da, respectively, calculated by PeakView software, and the removal debris difference was less than 0.01 Da. The compound contains glucoside and hydroxyl structures, which was consistent with the results of metabolic pathway deduction. After the compound information was imported into SciFinder, Reaxys, ChemSpider, and other online databases, no chemical structure matching M14 were found; thus., it was speculated that M14 is a new compound of *Epimedium.*

Pathway 1

Two kinds of monoglycoside components were found in this pathway, i.e., epimedoside C with glucoside bonded at the C-7 position, and baohuoside II with rhamnoside bonded to the C-3 site in the parent nucleus (desmethylicaritin). The secondary metabolism pattern diagram (Figure 1) shows that epimedoside D had a molecule of xyloside bonded at the C-3 site. It is speculated that there may be a compound bonded to xyloside in the parent nucleus. The molecular formula of this compound was then imported into PeakView software, and the first and second mass spectra of 15 species of *Epimedium* were extracted, combined with an online database, subjected to secondary fragmentation, and mass spectrometry cracking analysis. Consequently, no single xylosides and their methylated products were found. Epimedoside D has a glucoside attached to the C-7 site, while other xylosidic components are linked to the C-3 rhamnoside. These suggest that xylosides cannot be directly bonded to the parent nucleus, and the biochemical reaction can only occur after the C-7 hydroxyl group is combined with glucoside. It has been reported that the synthesis of the diglucoside compound was based on baohuoside II. However, only hexandraside E and epimedoside A were found in the diglucoside products with epimedoside C as the substrate, combined with the chemical structure of epimedoside D, which suggested that there might be diglucoside compounds containing C-3 xyloside C-7 glucoside. Finally, M1 and M2 were identified.

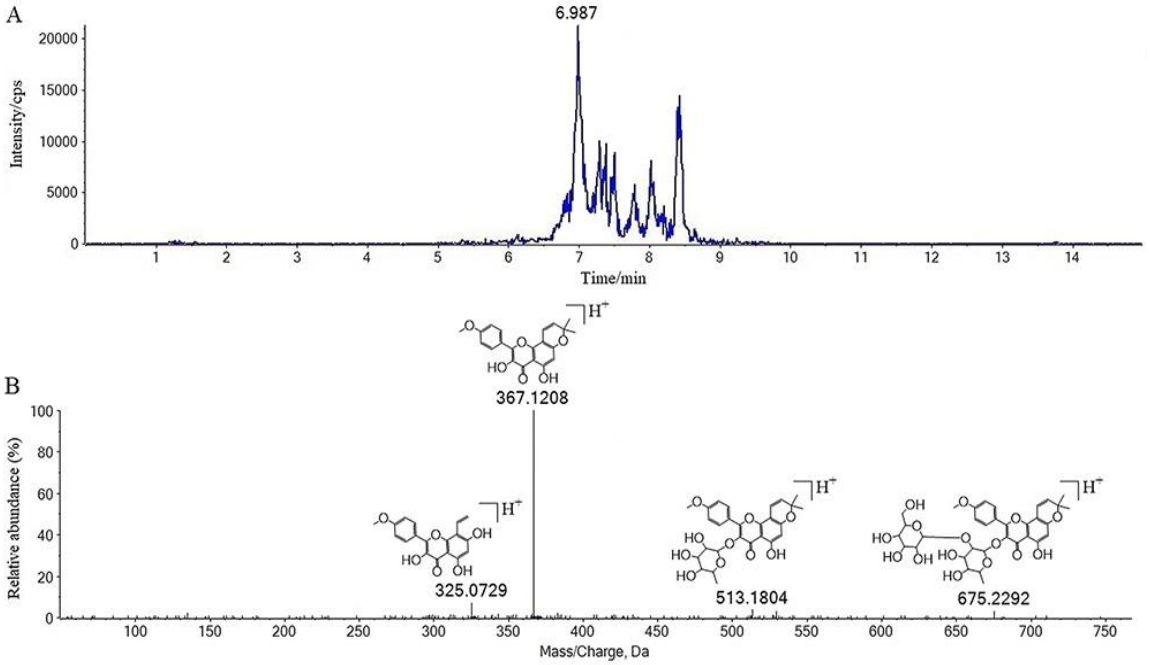

Fig. 19: Extraction ion diagram (A) and second-order fragmentation rule diagram (B) of compound M14.

Due to the existence of triglycosides and glucosinolate compounds in this pathway, we conducted corresponding analysis according to the laws of secondary metabolism; nevertheless, we did not identify any such component, which may be related to the *Epimedium* species, growth environment, metabolic accumulation, enzymes Activity, and other factors.

Pathway **2**

The secondary metabolic pattern of this pathway (Figure 2) showed that the parent nucleus (noricarliin) was obtained by adding one molecule of water to the double bond of isoprenyl at the C-8 site of desmethylicaritin. The structures of the other three components in the analysis path were the hydroxyglycosylation products at C-3, C-7, and hydroxymethylation at C-4'. It is speculated that this pathway was a result of a series of biochemical reactions under the catalysis of glycosyltransferase and methyltransferase in the parent nucleus that produce monoglycosides, diglycosides, and methylated products. Noricarliin's C-4' hydroxyl group was combined with methyl to form M3. The C-7, C-4' hydroxyl of parent nucleus bonded glucoside, and methyl can generate maohuoside A; so, it was speculated that there might be M4 that had not been oxidized before biosynthesis of maohuoside A.

Combined with PeakView software, secondary fragmentation, and mass spectrometry fragmentation rules, the derivation results of the two new compounds were also verified. In this pathway, no Xylose-containing monoglycosides and diglycosides other than wanepimedoside A were identified. Compared with the structure of desmethylicaritin, the double bond hydration addition occurred at the prenyl of parent nucleus C-8 site in this pathway, while the hydroxyl activity of C-3, C-7, C-4' site still exists. As a substrate, a variety of biochemical reactions may also occur under the catalysis of a transferase, and the chemical structure of four known components can support the above inference. Only two new compounds were identified from the 17 unknown components in this pathway, which may be related to the low substrate content and activity of key enzymes.

Pathway **3**

The secondary metabolic pattern of this pathway (Figure 3) showed that the parent nucleus (M5) was the product of the hydrogenation of the isoprenyl double bond at the C-8 site of desmethylicaritin. The entire pathway derivation process was consistent with pathway 2. The C-4' hydroxyl group of the parent nucleus was methylated to form M6. M7 was a methylated product of the parent nucleus bonded to one molecule of glucoside. M8 was a diglucoside compound of rhamnoside and glucoside, respectively bonded by C-3 and C-7 hydroxyl groups.

The derivation results of four new compounds were verified by PeakView software, secondary fragmentation, and mass spectrometry. In this pathway, no monoglucoside and diglycoside compounds containing xylose were identified. Among the 62 known flavonol components of *Epimedium*, only the presyl double bond at the C-8 site of ICARISID II was reduced, which was also the only known component in this pathway. In addition, there was a large amount of oxidation in plant reductases. Hydrogenation is a common type of reduction reaction; thus, it was speculated that the isopentenyl group at the C-8 site of desmethylicaritin might undergo double bond hydrogenation and form a series of metabolites. By analyzing the HRMS data, it was found that only M6 of the identified new compounds had ion peaks with C_4H_{10} (58.07 Da), while the other 3 compounds had fragments with C_4H_8 (56.06 Da) removed. The results suggest that there may be isomers of dihydroflavonols in this pathway. Also, the mass errors of this approach were less than 5 ppm, and the isotope distribution was also consistent, which eliminated the possibility of isotopes of other components.

Pathway **4**

Because the prenyl group at the C-8 site and the hydroxyl group at the C-7 site in the parent nucleus (M9) of this pathway form a ring, the hydroxyl group at the C-7 site can no longer be combined with the glycoside. Therefore, the only active groups that can be glycosylated is the C-3 hydroxyl group, while the C-4' hydroxyl group can only be methylated, and the C-5 hydroxyl group is not reactive. Figure 4 shows that cyclic cyclization of desmethylicaritin at the C-7 and C-8 sites can generate M9, after which, methylation at C-4' of M9 can generate anhydrocicaritin. Comparing the structure of ikarisoside E and acuminatin, we found that acuminatin is generated by the methylation at the C-4' site of ikarisoside E, while compared with M9, the C-7 and C-8 groups will be dehydrogenated again after cyclic cyclization to form a double bond, and

the C-3 position will bind a molecule of rhamnoside. It is speculated that the M9 group is dehydrogenated to form M10, and the C-4' site of M10 can be remethylated to form M11, and the glycosylation reaction can also occur on the C-3 hydroxyl of M10. The structural formulas of all compounds in the pathway were constructed by ChemDraw, and the compound information was imported into PeakView to extract and analyze samples data of 15 species of *Epimedium*. Combined with online databases, secondary fragments and mass spectrometry cleavage rule, it was identified that the C-3 hydroxyl group of M10 could also be combined with glucoside (M12), after which, the C-4' hydroxyl methylated (M13) is produced. In addition, one molecule of glucose or rhamnose monohydrate can be bonded to form two diglycoside compounds (M14, M15) based on the structure of ikarisoside E. As the C-7 hydroxyl group of the parent nucleus is blocked, the glycosylation activity of this group is lost. This pathway showed that no compound containing xylosides had been identified at the C-3 site, which was also consistent with the results derived from other pathways. This pathway does not address triglycoside compounds, because no component with more than 2 glycosides at the C-3 site had been found in the flavonol components of *Epimedium*.

Conclusions

In this study, 64 compound structures were deduced by constructing the secondary metabolism network of epimedium flavonol, combined with HRMS. Two new components and 12 new compounds were identified in 54 batches of *Epimedium* samples. The different compounds may be related to factors, such as the number of species samples, growing environment, harvest time, and processing methods, etc. By analyzing and summarizing the secondary metabolism of isoprenyl flavonol-containing components in *Epimedium*, we found that the hydroxymethylation of the parent nucleus (desmethylicaritin) occurs at the C-4' sites, the glucosylation usually occurs at the C-7 site, and glycosylation (rhamnoside) often occurs at the C-3 site, indicating that there are many similar biochemical reaction types in the flavonol biosynthesis pathway. This study also provides a scientific basis for the in-depth regulation of the epimedium secondary metabolism at the genetic and molecular levels. This research combined the characteristics of flavonoid metabolism and HRMS to deduce and verify the chemical structures of new components; yet, HRMS cannot ultimately determine the structure of compounds. The identification results still have the possibility of isomerization, chiral isomerization, cis-trans isomerization, etc. The structure of the compounds can only be finally determined by NMR spectroscopy after phytochemical separation. This research simplifies the long-time and cumbersome phytochemical separation steps, saves experimental costs, and provides a new idea and method for the analysis and identification of secondary metabolites with pharmacological activity.

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Conflict of Interest

Authors have no conflict of interest.

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